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Impact of estrogen replacement on ventricular myocyte contractile function and protein kinase B/Akt activation

Jun Ren,¹ Kadon K. Hintz,² Z. K. Fariba Roughead,³ Jinhong Duan,¹
Peter B. Colligan,² Bonnie H. Ren,¹ Kap J. Lee,⁴ and Huawei Zeng³

¹Division of Pharmaceutical Sciences, University of Wyoming College of Health Sciences, Laramie, Wyoming 82071-3375; ²University of North Dakota School of Medicine, Grand Forks 58203; ³United States Department of Agriculture, Grand Forks Human Nutrition Research Center, Agricultural Research Service, Grand Forks 58202; and ⁴Center for Biomedical Research, University of North Dakota School of Medicine, Grand Forks, North Dakota 58203

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Ren, Jun, Kadon K. Hintz, Z. K. Fariba Roughead, Jinhong Duan, Peter B. Colligan, Bonnie H. Ren, Kap J. Lee, and Huawei Zeng. Impact of estrogen replacement on ventricular myocyte contractile function and protein kinase B/Akt activation. *Am J Physiol Heart Circ Physiol* 284: H1800–H1807, 2003. First published January 16, 2003; 10.1152/ajpheart.00866.2002.—Women with functional ovaries have a lower cardiovascular risk than men and postmenopausal women. However, estrogen replacement therapy remains controversial. This study examined the effect of ovarian hormone deficiency and estrogen replacement on ventricular myocyte contractile function and PKB/Akt activation. Nulliparous female rats were subjected to bilateral ovariectomy (Ovx) or sham operation (sham). A subgroup of Ovx rats received estrogen (E₂) replacement (40 µg·kg⁻¹·day⁻¹) for 8 weeks. Mechanical and intracellular Ca²⁺ properties were evaluated including peak shortening (PS), time to PS (TPS), time to 90% relengthening (TR₉₀), maximal velocity of shortening/relengthening (±dL/dt), fura 2 fluorescence intensity (FFI), and decay rate. Levels of sarco(endo)plasmic reticulum Ca²⁺-ATPase (SERCA2a), phospholamban (PLB), and Akt were assessed by Western blot. Ovx promoted body weight gain associated with reduced serum E₂ and uterine weight, all of which were abolished by E₂. Ovx depressed PS and ±dL/dt, prolonged TPS, TR₉₀, and decay rate, and enhanced resting FFI, all of which, with the exception of TPS, were restored by E₂. Ovx did not alter the levels of SERCA2a, PLB, and total Akt, but significantly reduced Akt activation [phosphorylated Akt (pAkt)], pAkt/Akt, and the SERCA2a-to-PLB ratio. These alterations in protein expression were restored by E₂. E₂ enhanced PS and +dL/dt in vitro, which was abolished by the E₂ receptor antagonist ICI-182780. Ovx reduced myocyte Ca²⁺ responsiveness and lessened stimulating frequency-induced decline in PS, both ablated by E₂. These data suggest that mechanical and protein functions of ventricular myocytes are directly regulated by E₂.

ovariectomy; cardiac myocyte; contraction, intracellular Ca²⁺

GENDER GAP IN CARDIOVASCULAR diseases has long been recognized and has led to considerable speculation regarding the underlying etiology (29). The fact that women have a lower incidence of cardiovascular dis-

ease before menopause but lose this gender advantage with the onset of menopause indicates that ovarian hormones, primarily estrogen (E₂), play a pivotal role in reducing risk for cardiovascular disease (8, 11, 34). Compelling evidence has confirmed the close relationship between levels of E₂ and heart function, supported by both clinical and experimental evidence that E₂ replacement therapy in postmenopausal women may ameliorate cardiac risk, although this notion has been challenged recently (7, 13, 19, 30, 31). Although the beneficial effect of E₂ is believed to be due to reduced low-density lipoprotein oxidation, decreased oxidative stress, as well as enhanced high-density lipoproteins (15, 31), recent clinical trials (17, 28) in women with coronary heart diseases did not reveal any beneficial effects on overall heart condition with E₂ replacement therapy. Thus the cardioprotective effect of estrogens appears to be more complicated than originally thought and requires more research. The fact that the correlation between E₂ and lipid profiles in hearts may not be used to simply predict cardiac function may suggest that E₂ possesses other effects on hearts. E₂ may directly regulate cardiac function and is responsible for the gender difference in myocardial morphology, function, and prevalence of cardiac risk (4, 25, 27, 30). Ovariectomy during pre- and postpubertal periods has been shown to lead to decreased cardiac output, peak systolic pressure, and ejection fraction associated with reduced myosin ATPase activity and myosin isoenzyme shift (V₁ to V₃) (4), which can be prevented by E₂ replacement therapy (26). In addition, E₂ has also been shown to promote nitric oxide production and improve insulin resistance, which may affect cardiac function indirectly (18, 32).

With this background, it is logical to speculate that the ovarian hormones, especially E₂, play a physiological role in ventricular pumping function. However, the direct impact of E₂ deficiency reminiscent of menopause and E₂ replacement on cardiac contractile func-

Address for reprint requests and other correspondence: J. Ren, Div. of Pharmaceutical Science, Univ. of Wyoming College of Health Sciences, Laramie, WY 82071-3375 (E-mail: jren@uwyo.edu).

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tion at the ventricular myocyte level has not been elucidated. This study was designed to determine whether the cardiac mechanical properties at the ventricular myocyte level and certain key cardiac regulatory proteins, such as sarco(endo)plasmic reticulum Ca^{2+} -ATPase (SERCA2a), phospholamban (PLB), and PKB/Akt were affected by E_2 deficiency and, subsequently, E_2 replacement.

MATERIALS AND METHODS

Animals and E_2 replacement. All animal procedures were approved by the University of North Dakota and University of Wyoming Animal Care and Use Committees. In brief, 70-day-old mature nulliparous female Sprague-Dawley rats (National Cancer Institute; Bethesda, MD) weighing 150–175 g were assigned to weight-paired ovariectomy (Ovx) or sham-operated (sham) groups. For the Ovx group, after anesthesia, the ovaries were exteriorized, ligated, and removed via bilateral paralumbar incisions, which were then closed with sterile sutures. The sham procedure consisted of anesthesia, visualization of the ovaries through incisions into the abdominal cavity, and closure of the wounds. One week after the surgery, a subgroup of the Ovx rats were assigned to the E_2 replacement group receiving daily intraperitoneal injection of 17β -estradiol (40 $\mu\text{g}/\text{kg}$ in 100 μl cottonseed oil). The control group received vehicle only. Treatment lasted for 8 wk. At the time of death, adequacy of Ovx was determined by absence of ovarian tissue and marked atrophy of the uterus (measurement of uterine weight) in female rats. Serum 17β -estradiol was measured by using an enzyme-linked immunoassay kit (Cayman Chemical; Ann Arbor, MI).

Cell isolation procedures. Ventricular myocytes were enzymatically isolated as described (23), with modifications. In brief, hearts were removed and perfused (at 37°C) with Krebs-Henseleit bicarbonate (KHB) buffer. Hearts were perfused with Ca^{2+} -free KHB buffer containing 223 U/ml collagenase (Worthington Biochemical; Freehold, NJ) for 16 min. After perfusion, ventricles were removed, minced, and filtered through a nylon mesh (300 μm). Myocytes were resuspended in a sterile-filtered, Ca^{2+} -free Tyrode buffer containing (in mM) 131 NaCl, 4 KCl, 1 MgCl_2 , 10 HEPES, and 10 glucose, supplemented with 2% bovine serum albumin, with a pH of 7.4 at 37°C . Extracellular Ca^{2+} was slowly added back to 1.25 mM. Freshly isolated myocytes from sham or Ovx (with or without E_2) rats were used within 8 h of isolation. In a separate experiment, ventricular myocytes from adult female rats were cultured in a serum-free medium (medium 199; Sigma) with or without supplementation of E_2 (10^{-9} M) or the E_2 receptor antagonist ICI-182780 (10^{-8} M; Tocris Cookson, Ellisville, MO) for 24 h before use (20). ICI-182780 was dissolved in DMSO, the final concentration of which was $<0.01\%$, and did not affect myocyte mechanics.

Cell shortening/relengthening. Mechanical properties of ventricular myocytes were assessed by using a video-based MyoCam system (IonOptix; Milton, MA) (23). In brief, cells were superfused with a buffer containing (in mM) 131 NaCl, 4 KCl, 1 CaCl_2 , 1 MgCl_2 , 10 glucose, and 10 HEPES at pH 7.4 and were field stimulated at 0.5 Hz. Myocytes were displayed on the computer monitor by using an IonOptix MyoCam camera, which rapidly scans the image area at every 8.3 ms, such that the amplitude and velocity of shortening/relengthening is recorded with good fidelity.

Intracellular fluorescence measurement. Myocytes were loaded with fura 2-AM (0.5 μM) for 15 min and fluorescence measurements were recorded with a dual-excitation fluorescence photomultiplier system (Ionoptix) (23). Myocytes were

imaged through an Olympus fluor $\times 40$ oil objective and exposed to light emitted by a 75-W lamp and passed through either a 360- or 380-nm filter (bandwidths were ± 15 nm), while being field stimulated to contract at 0.5 Hz. Fluorescence emissions were detected between 480 and 520 nm after cells were first illuminated at 360 nm for 0.5 s and then at 380 nm for the duration of the recording protocol (333 Hz sampling rate). The 360-nm excitation scan was repeated at the end of the protocol and qualitative changes in intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) were inferred from the ratio of the fluorescence intensity at two wavelengths.

Western analysis of SERCA2, PLB, and phosphorylated Akt. Membrane proteins from the left ventricular myocardium of each heart were isolated as described (35). Freshly dissected hearts were homogenized and centrifuged at 1,000 g for 10 min. The supernatants were then centrifuged at 70,000 g for 30 min at 4°C . The 100,000-g pellets were cellular membrane fractions and were used for immunoblotting of SERCA2, PLB, and Akt [both total and phosphorylated (pAkt)]. We confirmed that these membrane fractions did not contain any detectable collagens. Membrane proteins (50 $\mu\text{g}/\text{lane}$) were separated on 7% (SERCA2a and Akt) or 15% (PLB) SDS-polyacrylamide gels in a minigel apparatus (Mini-PROTEAN II; Bio-Rad) and transferred to polyvinylidene difluoride membranes. The membranes were blocked (4% Block Ace; Dainippon Pharmaceutical, Osaka, Japan) and then incubated with anti-SERCA2 (1:1,000 dilution), anti-PLB (1:1,000), anti-Akt, and anti-pAkt (1:1,000) antibodies [monoclonal antibodies to SERCA2a (A7R5) and PLB (2D12) were kindly provided by Dr. Larry Jones, Indiana University School of Medicine]. Anti-Akt and anti-pAkt antibodies were obtained from Upstate Biotechnology (Lake Placid, NY). The antigens were detected by enhanced chemiluminescence (ECL Western blotting detection kit, Amersham) with peroxidase-linked anti-mouse (SERCA2a and PLB), anti-rabbit (pAkt), or anti-sheep (Akt) IgG (1:5,000 dilution). After the immunoblotting, the film was scanned and the intensity of immunoblot bands was detected with a Bio-Rad Calibrated Densitometer (model GS-800).

Statistical analyses. For each experimental series, data are presented as means \pm SE. Statistical significance ($P < 0.05$) for each variable was estimated by ANOVA or *t*-test where appropriate.

RESULTS

General features of the experimental animals. Eight weeks after operation, rats from the Ovx group displayed significantly elevated body weight gain and reduced serum E_2 levels and uterine weight compared with the sham-operated animals. Interestingly, these Ovx-induced alterations were ablated with E_2 replacement therapy. The liver and kidney but not the heart weights were significantly heavier in ovariectomized rats with or without E_2 replacement; however, the organ-to-body weight ratio was comparable in all three groups studied (Table 1).

Effect of Ovx and E_2 replacement on myocyte shortening. The resting cell length (CL) was 139 ± 4 , 124 ± 4 , and 111 ± 3 μm in sham, Ovx and Ovx + E_2 groups, respectively ($n = 191$ – 192 cells from 5–6 rats per group). Neither Ovx nor E_2 replacement had any overt effects on cell phenotype. The cell shape and presence of distinct striations were comparable in all three groups studied (data not shown). Representative traces

Table 1. General features of experimental animals

	Sham	Ovx	Ovx + E ₂
<i>n</i>	6	7	6
Body wt, g	275.2 ± 6.1	318.3 ± 15.8*	293.5 ± 9.9
Heart wt, g	1.19 ± 0.04	1.35 ± 0.09	1.27 ± 0.06
Heart wt/body wt, mg/g	4.30 ± 0.09	4.26 ± 0.24	4.34 ± 0.19
Liver wt, g	8.28 ± 0.38	9.87 ± 0.64*	9.21 ± 0.19*
Liver wt/body wt, mg/g	30.2 ± 1.8	31.3 ± 2.1	31.6 ± 1.4
Kidney wt, g	1.77 ± 0.04	1.99 ± 0.07*	2.07 ± 0.10*
Kidney/body wt, mg/g	6.44 ± 0.18	6.34 ± 0.35	7.07 ± 0.30
Uterine weight, g	0.66 ± 0.11	0.17 ± 0.03*	0.49 ± 0.09
Serum estrogen, pg/ml	72.4 ± 20.9	6.0 ± 2.1*	66.7 ± 19.3

Values are means ± SE; *n* = number of animals. Ovx, ovariectomy; E₂, estrogen replacement. **P* < 0.05 vs. sham group.

are shown in Fig. 1A, depicting typical contractile profiles of ventricular myocytes from sham, Ovx and Ovx + E₂ groups. Figure 1, B–F, demonstrates that myocytes from Ovx animals exhibited reduced peak shortening (PS) and maximal velocity of shortening and relengthening ($\pm dL/dt$) associated with prolonged

duration of shortening (TPS) and relengthening (TR₉₀). Interestingly, all mechanical alterations due to Ovx (with the exception of TPS) were restored by E₂ replacement.

Effect of Ovx and E₂ replacement on intracellular Ca²⁺ transients. To determine whether the mechanical effect of either Ovx or E₂ replacement on ventricular myocytes was due to changes in intracellular Ca²⁺ handling, intracellular Ca²⁺ homeostasis in ventricular myocytes was assessed with fura 2 fluorescent microscopy. The fluorescence decay was fit by a single exponential equation, and the time constant (τ) was calculated as a measure of the rate of decline of free cytoplasmic Ca²⁺. The fluorescence measurements revealed that myocytes from the Ovx group displayed significantly elevated resting intracellular Ca²⁺ level and slowed intracellular Ca²⁺ clearing (longer τ), consistent with our previous findings (12). Both of these Ovx-induced changes in intracellular Ca²⁺ handling were restored by E₂ replacement. The increase of [Ca²⁺]_i (Δ [Ca²⁺]_i) in response to electrical stimuli was

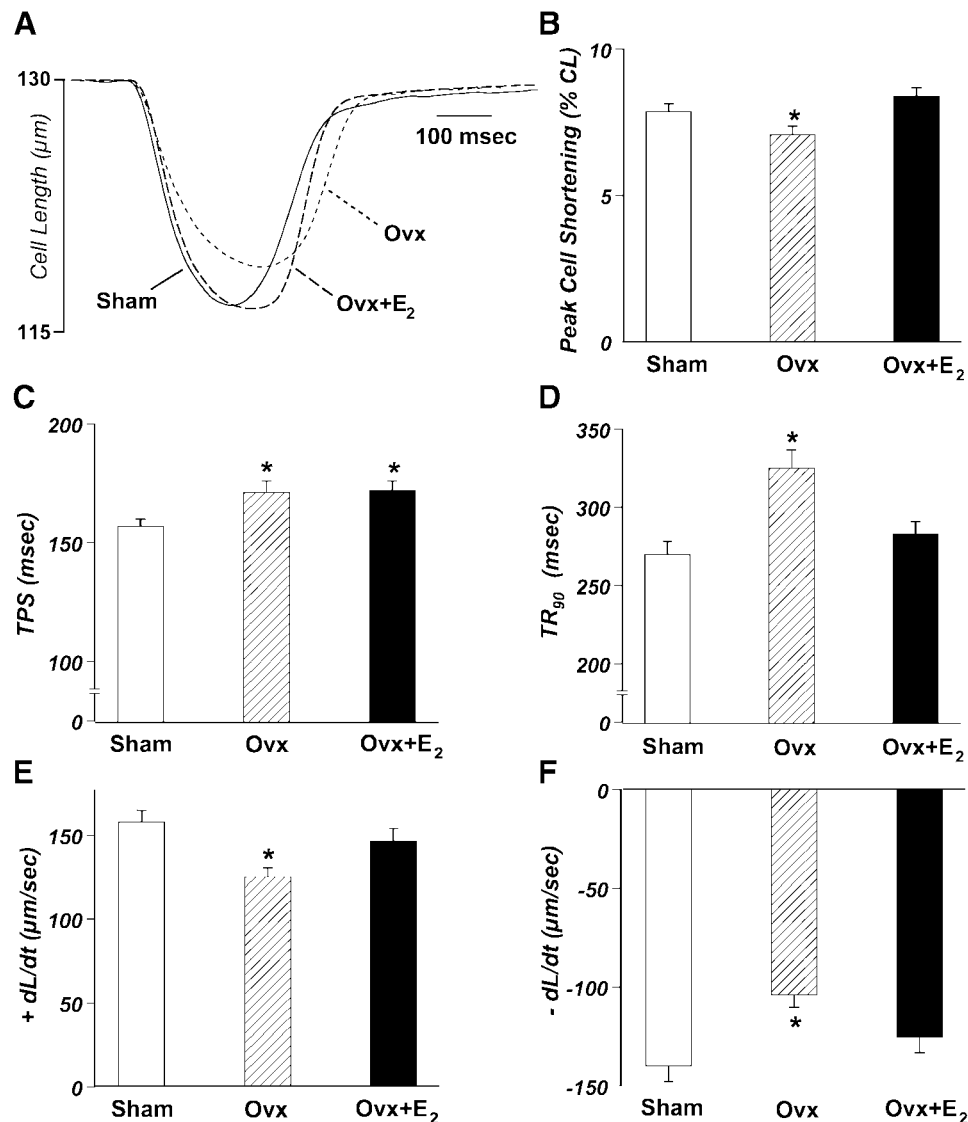


Fig. 1. Mechanical properties of ventricular myocytes from sham, ovariectomized (Ovx), and Ovx with estrogen replacement groups (Ovx + E₂). A: representative traces depicting cell shortening in myocytes from sham, Ovx, and Ovx + E₂ groups. B: peak cell shortening (PS) amplitude as a percentage of resting cell length (CL). C: duration of shortening (TPS). D: duration of relengthening (TR₉₀). E: maximum velocity of shortening ($+dL/dt$). F: maximal velocity of relengthening ($-dL/dt$). Values are means ± SE, *n* = 148 cells from 5–6 animals per group. **P* < 0.05 vs. sham group.

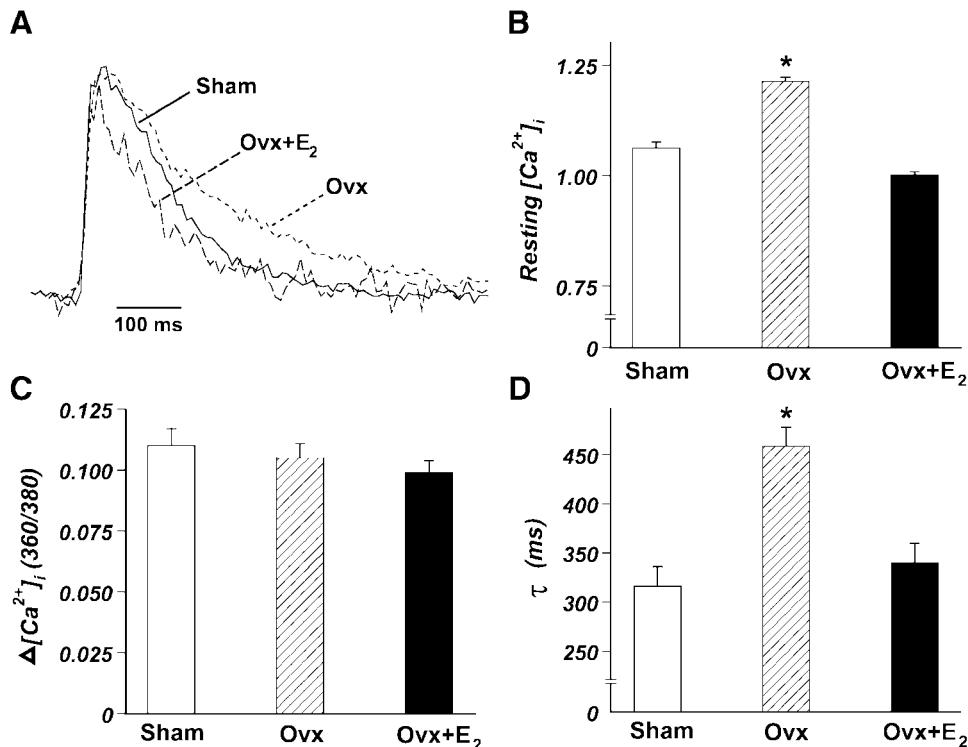


Fig. 2. Intracellular Ca^{2+} handling properties in myocytes from sham, Ovx, and Ovx + E_2 groups. **A**: representative traces depicting intracellular Ca^{2+} transients in myocytes from sham, Ovx, and Ovx + E_2 groups. **B**: resting intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) levels. **C**: increases in $[Ca^{2+}]_i$ transients in response to electrical stimuli ($\Delta[Ca^{2+}]_i$). **D**: intracellular Ca^{2+} transient decay rate (τ). Values are means \pm SE, $n = 95$ cells from 5–6 animals per group. * $P < 0.05$ vs. sham group.

similar among all three groups studied (Fig. 2). These results revealed potentially compromised intracellular Ca^{2+} handling in hearts from Ovx rats and the protective effect of E_2 replacement. Myocyte shortening was also recorded from fura 2-loaded cells but was used for qualitative comparisons only, to avoid potential effects on contraction from intracellular Ca^{2+} buffering by fura 2.

Effect of Ovx and E_2 replacement on myocyte shortening with increased extracellular Ca^{2+} . The effect of extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$) on myocyte shortening was examined in myocytes from sham, Ovx, and Ovx + E_2 groups. Increases in $[Ca^{2+}]_o$ from 0.5 mM up to 3.0 mM resulted in a positive staircase in the amplitude of myocyte shortening in all groups, as expected. However, the PS amplitude was significantly less in myocytes from the Ovx group at $[Ca^{2+}]_o$ between 1.0 and 3.0 mM compared with those from the sham group. The discrepancy in PS amplitude between Ovx and sham groups was abolished by E_2 replacement (Fig. 3), suggesting that E_2 may preferentially affect Ca^{2+} responsiveness in ventricular myocytes.

Effect of Ovx and E_2 replacement on myocyte shortening with increasing stimulation frequency. To look for possible derangement of cardiac excitation-contraction coupling, the stimulating frequency was increased up to 5 Hz (300 beat/min) and steady-state PS was recorded. Cells were initially stimulated to contract at 0.5 Hz for 5 min before the frequency study was commenced. Steady state was normally reached five to six beats after a change in frequency. All recordings were normalized to PS at 0.1 Hz (as 100%) of the same myocyte. Figure 4 shows a negative staircase in PS

with increased frequency in myocytes from all animal groups. However, myocytes from the Ovx group exhibited a lesser reduction in PS with increasing stimulus frequency compared with the sham or Ovx + E_2 groups, indicating a change of sarcoplasmic reticulum Ca^{2+} replenishing ability with ovarian hormone deficiency.

Effect of the E_2 antagonist ICI-182780 on E_2 -induced myocyte mechanical response. To examine if short-term exposure of E_2 possesses any cardiac mechanical effect

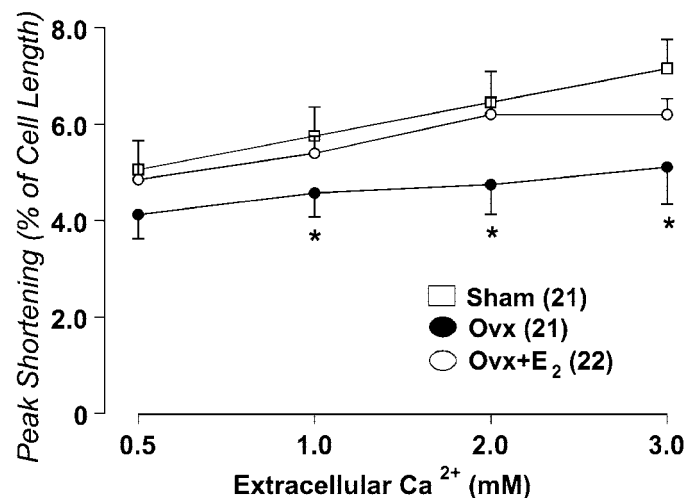


Fig. 3. Effects of increase in extracellular Ca^{2+} concentration (0.5–3.0 mM) on PS in myocytes from sham, Ovx, and Ovx + E_2 groups (5–6 animals each). PS was presented as percent change from resting CL. Values are means \pm SE; sample size is given in parentheses. * $P < 0.05$ vs. sham group.

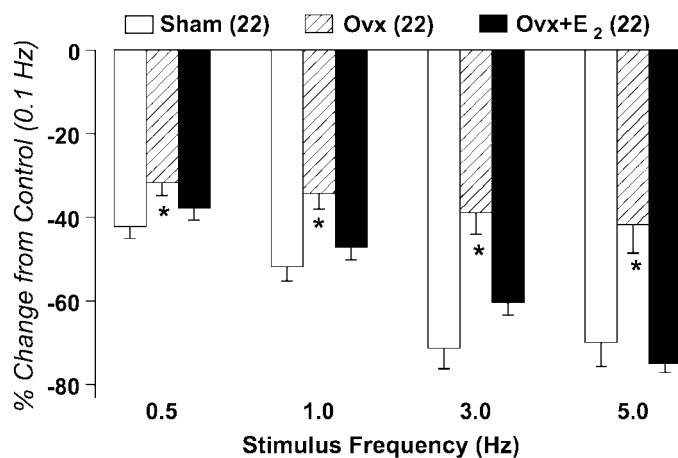


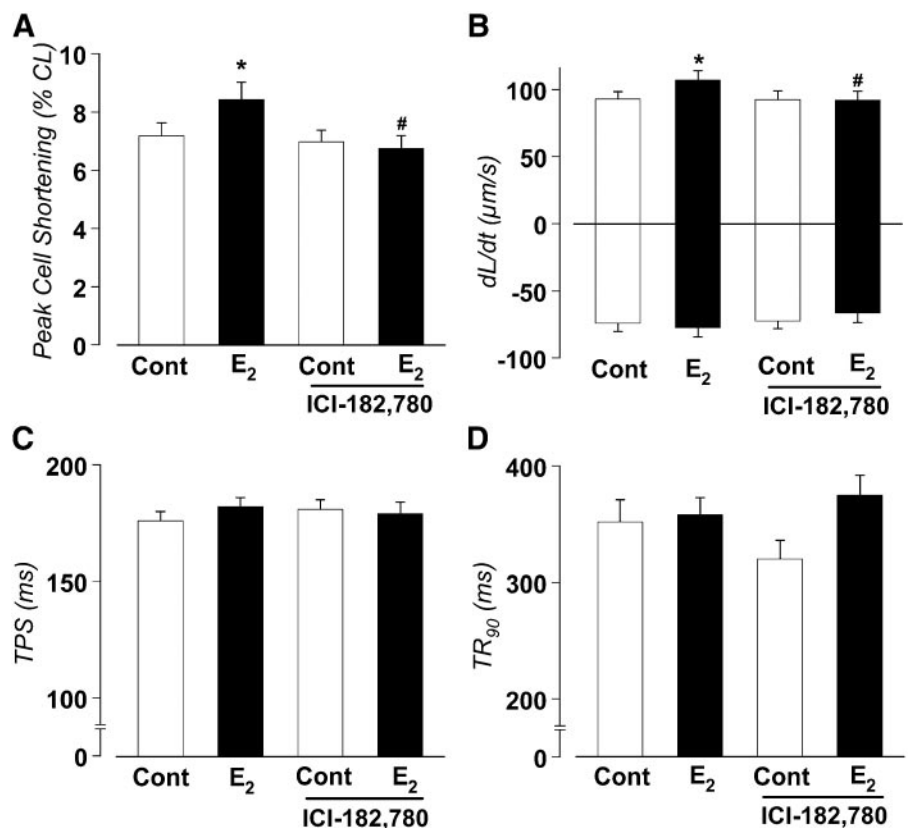
Fig. 4. Effects of increased stimulus frequency (0.1–5.0 Hz) on myocyte PS amplitude in myocytes from sham, Ovx, and Ovx + E₂ groups (5–6 animals each). PS was presented as percent change from respective PS obtained at 0.1 Hz. Values are means \pm SE; sample size is given in parentheses. * P < 0.05 vs. sham group.

through its membrane receptor, ventricular myocytes from normal adult female rats were maintained in a control or an E₂ (10⁻⁹ M) supplemented medium with or without the high-affinity E₂ receptor antagonist ICI-182780 (10⁻⁸ M, 20) for 24 h. The resting CL was 156 ± 2 μ m (n = 186 cells). Neither E₂ nor ICI-182780 elicited any overt effect on cell shape, resting CL, and the presence of striations. Consistent with the data observed previously from in vivo study, ventricular myocytes maintained in E₂-containing medium exhib-

ited enhanced PS and +dL/dt, which were abolished by the E₂ receptor antagonist ICI-182780. None of the other mechanical indices (-dL/dt, TPS, and TR₉₀) were affected by E₂ or ICI-182780 (Fig. 5). These data suggested that E₂ may directly exert cardiac mechanical effect via its membrane receptor, the E₂ receptor.

Western blotting of SERCA2, PLB, and PKB/pAkt. Alterations of cardiac mechanical properties and intracellular Ca²⁺ homeostasis may be a reflection of changes in certain regulatory proteins for intracellular Ca²⁺ handling and myocyte function such as SERCA, PLB, and PKB/Akt activation (1, 14, 16). To examine the role of these proteins in the altered mechanical and intracellular Ca²⁺ properties under Ovx or E₂ replacement conditions, protein levels of SERCA2a, PLB, and PKB/Akt from hearts of all three groups were measured by Western blot. As shown in Fig. 6A, neither Ovx nor E₂ replacement affects the total Akt level. However, Akt activation, presented as either the absolute phosphorylated Akt (pAkt) level or as a percentage of total unphosphorylated Akt (pAkt-to-Akt ratio), was significantly reduced in the Ovx group and restored by E₂ replacement. Our further immunoblotting analysis revealed that SERCA2a and PLB protein levels were not significantly different among all three groups tested. However, the SERCA2a-to-PLB ratio was significantly reduced in the Ovx group compared with the sham group, which often indicates reduced cardiac contractile function (14). This Ovx-induced reduction in SERCA2a-to-PLB ratio was restored by E₂ replacement (Fig. 6B). The reduced SERCA2a-to-PLB ratio

Fig. 5. Mechanical properties of adult female rat ventricular myocytes maintained for 24 h in control (Cont) or E₂ 10⁻⁹ M medium with or with the E₂ receptor antagonist ICI-182780 (10⁻⁸ M). A: PS as a percentage of resting CL; B: \pm dL/dt (in μ m/s); C: TPS (in ms); D: TR₉₀ (in ms). Values are means \pm SE; n = 46–48 cells. * P < 0.05 vs. Cont group; # P < 0.05 vs. E₂ group.



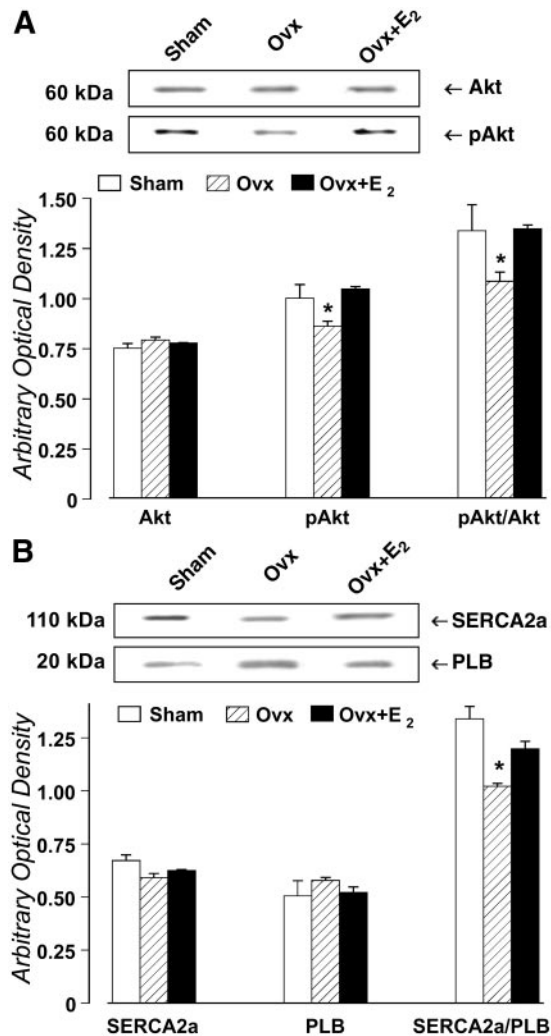


Fig. 6. Western blot analysis of Akt, Akt phosphorylation (pAkt), sarco(endo)plasmic reticulum Ca²⁺-ATPase (SERCA2a), and phospholamban (PLB) in whole heart homogenates from sham, Ovx, and Ovx + E₂ groups. A: arbitrary optical density of Akt and pAkt as well as the pAkt-to-Akt ratio. Insets show representative blot using anti-Akt and anti-pAkt antibodies. B: arbitrary optical density of SERCA2a and PLB as well as the SERCA2a-to-PLB ratio. Insets show representative blots using anti-SERCA2a and anti-PLB antibodies. Values are means \pm SE, $n = 3$. * $P < 0.05$ vs. sham group.

was consistent with the functional data of depressed ventricular contractility, slowed intracellular Ca²⁺ removal, and prolonged duration of contraction (14).

DISCUSSION

The present study demonstrated, for the first time, that E₂ deficiency due to Ovx directly affects ventricular myocyte contractile function and intracellular Ca²⁺ handling associated with reduction in PKB/Akt activation and SERCA2a-to-PLB ratio. Our results revealed decreased peak myocyte shortening, reduced maximal velocities of shortening/relengthening, and markedly prolonged duration of shortening and relengthening in myocytes from ovariectomized rat hearts. These mechanical abnormalities may be underscored by altered intracellular Ca²⁺ homeostasis, shown as slowed intra-

cellular Ca²⁺ clearing and elevated resting intracellular Ca²⁺ levels. Our immunostaining study also indicated that the altered mechanical and intracellular Ca²⁺ homeostasis may be associated with a reduced ratio of the main Ca²⁺-regulating protein SERCA/PLB under E₂ deficiency. We also found reduced activation of Akt, a protein kinase believed to be directly regulated by E₂ (3). Interestingly, the E₂ deficiency-induced cardiac mechanical alterations (except prolonged TPS) were significantly restored with daily E₂ replacement therapy, supporting an essential role of ovarian hormones, primarily E₂, in the regulation of cardiac contractile function. Our in vitro E₂ exposure study suggested that the E₂-induced cardiac mechanical effects may be mediated through its specific membrane receptor.

Our study confirmed earlier observations that Ovx increased body weight gain and hepatic as well as renal hypertrophy (5, 25, 26). The change in body weight caused by Ovx is not fully understood, although loss of E₂-regulated metabolic/anabolic action on lipid profile may play a role (24). A recent study also suggested that the body weight gain after Ovx may be accompanied by an increased leptin level, which was eliminated by E₂ replacement therapy (5). The experimental model of E₂ deficiency is verified by reduced serum E₂ levels and uterine weight and the fact that E₂ replacement restored both serum E₂ levels and uterine weight.

In our study, Ovx imposed significant changes on myocyte mechanics (depressed PS and $\pm dL/dt$; prolonged TPS and TR₉₀). Moreover, myocytes from ovariectomized rats exhibited elevated resting intracellular Ca²⁺ levels and slowed intracellular Ca²⁺ clearing, indicative of altered intracellular Ca²⁺ handling. These findings are somewhat consistent with our earlier observations (12). Different myocardial mechanical function has been documented between males and females, mostly characterized by shorter contraction and faster tension development/decline associated with comparable peak tension development in females (4, 6). It is believed that the ovarian hormone-related disparity in contractile protein expression/function is responsible for the mechanical differences. This notion is supported by our in vitro finding that E₂ directly enhanced PS and maximal velocity of shortening, likely through specific E₂ receptors. E₂ receptors are present on a variety of cell types including ventricular myocytes. E₂ may modulate gene expression in cardiac myocytes, indicating that heart is a target for sex steroid actions (10). Deficiency in E₂ may lead to abnormalities in cardiac excitability and enhanced propensity for cardiac dysfunctions through an increase in the number of Ca²⁺ channels. Ovariectomy was shown to upregulate the L-type Ca²⁺ channel density (21), which may be related to elevated resting intracellular Ca²⁺ level observed in our study. It is worth mentioning that reduced L-type Ca²⁺ channel density has also been reported after Ovx (2). The mechanisms involved in alteration of intracellular Ca²⁺ entry/extrusion after Ovx remain unclear but may play a role in altered intracellular Ca²⁺ handling leading to elevated resting

intracellular Ca^{2+} and slowed intracellular Ca^{2+} clearing. Ovarian hormones (e.g., E_2 , progesterone) alter myocardial contractile function such as myofilament Ca^{2+} sensitivity without significant change in the maximum force development (33). This is also reflected in the PS-stimulus frequency response. The lessened reduction of PS in response to elevated stimulus frequency in Ovx myocytes may indicate a more efficient intracellular Ca^{2+} replenishing ability from the sarcoplasmic reticulum, which is brought back to its original level with E_2 replacement. Finally, the fact that not all mechanical indices were equally affected by 24-h treatment of E_2 indicates disparity in the responsiveness (including duration requirement) of Ca^{2+} regulating proteins to the hormone.

The reduced Akt activation in myocytes from ovariectomized rats and the ability of E_2 replacement to restore Akt activation coincides with the mechanical as well as intracellular Ca^{2+} handling data, suggesting that Akt may play a role in E_2 -regulated cardiac function. Linkage of the Akt signaling cascade to the modulation of cardiac contractile function is not fully clear. Direct evidence is not available regarding the cardiac contractile response of myocytes to Akt. However, observations from two independent groups have provided compelling evidence on the functional role of Akt. Enhanced myocardial contraction in conjunction with increased Ca^{2+} release from ryanodine receptor Ca^{2+} -release channels, Ca^{2+} sparks, and electrically stimulated Ca^{2+} transients was reported to be paralleled with an augmented phosphatidylinositol 3-kinase (PI3-kinase)-dependent phosphorylation of Akt (21). In vivo gene transfer of constitutively active Akt mutant in a rat model of cardiac ischemia-reperfusion injury has led to dramatically improved cardiac function (16). In contrast, dominant negative Akt, which blocks Akt activation, accelerated hypoxia-induced cardiomyocyte dysfunction and death (16). The potential cardiac contractile effect of Akt may also be evidenced by the cardiac contractile response induced by PI3-kinase and its downstream signaling of phospholipase C (9, 22). It may be speculated that Akt represents an important control point determining not only cardiomyocyte survival, but also function, under various statuses of E_2 . Elucidation of the precise role of Akt should provide invaluable information regarding new drug development for heart diseases.

Another interesting observation of the present study is that the SERCA2a-to-PLB ratio declined with Ovx but was restored with E_2 replacement, again coinciding with the mechanical changes under both conditions. SERCA contributes to ~92% of the cytosolic Ca^{2+} removal workload in rat hearts, whereas PLB is the main inhibitor of SERCA, acting to keep SERCA function "in check" (1). An increase in PLB-to-SERCA2 ratio reduces the SERCA Ca^{2+} affinity and activity, leading to prolonged relaxation and a reduced contractility. On the other hand, a decreased PLB-to-SERCA2 ratio improves the cardiac Ca^{2+} cycling and pumping function (14). Although we did not observe significant alteration in either SERCA2a or PLB alone in E_2 deficiency or

replacement groups, we observed a reduced SERCA2a-to-PLB ratio in Ovx hearts, consistent with the prolonged τ/TR_{90} and reduced PS. More importantly, E_2 replacement restored the SERCA2a-to-PLB ratio, which may underscore the effect of E_2 on Ovx-induced prolonged relaxation (TR_{90}) and reduced PS and $\pm dL/dt$. The significance of the PLB-to-SERCA2 ratio on myocardial contractile regulation has been demonstrated in rodent models with variable expression levels of PLB and SERCA (14). It is not clear at this point why prolonged TPS resulting from Ovx was not restored with E_2 replacement. One speculation is that the contractile (shortening) phase may be regulated concurrently by other cardiac contractile mechanism(s) independent of E_2 or its downstream signaling molecules.

Taken together, our experimental findings suggest that E_2 plays a significant role in the regulation of cardiac contractile function at the level of ventricular myocytes, and E_2 replacement may have potential protective effects against ovarian hormone deficiency-induced alteration of cardiac contractile function.

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